

# “*Candidatus* Hepatobacter penaei,” an Intracellular Pathogenic Enteric Bacterium in the Hepatopancreas of the Marine Shrimp *Penaeus vannamei* (Crustacea: Decapoda)

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**The bacteria that cause necrotizing hepatopancreatitis in *Penaeus vannamei* adversely affect penaeid shrimp cultured in the western hemisphere. 16S rRNA and gyrase B gene analyses determined the taxonomic position of these bacteria. The name “*Candidatus* Hepatobacter penaei” is proposed for these pathogenic bacteria, which are members of the *Rickettsiales* order.**

Infection with necrotizing hepatopancreatitis bacteria (NHP-B) and the resultant subsequent deaths of marine shrimp have been well documented over the past 2 decades, and the bacteria are considered an economically significant pathogen of *Penaeus vannamei*. Although the bacterium has been well characterized, *in vitro* attempts to culture the causative organism have remained unsuccessful (1–6). The 16S rRNA and gyrase B gene sequences were used to elucidate the phylogenetic position of NHP-B more clearly, placing these bacteria within the order *Rickettsiales*. The proposed classification of NHP-B is “*Candidatus* Hepatobacter penaei.”

The hepatopancreata (HPs) from *P. vannamei* originating from an NHP-B outbreak in Mexico in 2011 were confirmed to be infected by PCR (1, 7) and semipurified (8, 9). Transmission electron microscopy confirmed the two morphological stages. Both rod and helical forms, including the presence of eight periplasmic flagella projecting from the basal end of a mature bacterium, were visualized (2, 8). DNA was extracted from the semipurified preparation, and PCR amplification of the 16S rRNA and gyrase B genes was performed with previously published universal primers and cycling parameters (10, 11). The products were cloned, and three clones from each gene were sequenced. Alignment was done with CLUSTAL W (12), and Bayesian inference analysis was performed with MRBAYES with one cold chain and three heated chains each, running for 500,000 generations (13). Phylogenetic analyses were conducted using MEGA version 5 (14).

Comparison of the reference sequence of NHP-B (accession number U65509) to the 16S rRNA gene sequence from this study (accession number JX981946) showed two mismatches in the 1,415-nucleotide sequences. In positions 230 and 990 of the reference sequence, the nucleotide is adenine (A), but in both instances, guanine (G) replaced adenine in the sequence generated for this study.

As had been determined previously by Loy et al. (6) using the 16S rRNA gene, this bacterium is phylogenetically associated with the order *Rickettsiales*. Recent nucleotide sequence additions from this order of bacteria to public databases refines the placement of the 16S rRNA sequence in the genera classified as “incertae sedis,” with low support branching probability (64.76%) in the family *Holospiraceae* (Fig. 1A) (15–19). Both *Holospira* spp. and *Caedibacter* spp. are intracellular bacteria in paramecia. *Holospira* species infect the nuclei of paramecia and are generally considered pathogenic to their hosts, while most species of *Caedibacter* are

toxic to specific sensitive strains of paramecia by conferring a killer trait or a mate killer trait upon their host cell (15, 16). Many bacterial species in the order *Rickettsiales* have been found associated with invertebrates (20), and most members of the *Rickettsiales* order are associated with freshwater or damp terrestrial environments (21).

The gyrase B gene sequence phylogenetic analysis of 1,259 bp (Fig. 1B) further supports the placement of NHP-B in the *Rickettsiales* order. On the basis of fully sequenced genomes deposited in public databases, the most closely related species is *Anaplasma phagocytophilum*, a tick-borne pathogen of mammals (22). The bacterium described in this study is the first example of a member of the *Rickettsiales* order that is from a marine environment and is pathogenic to an invertebrate that is not only an arthropod but also a crustacean.

Possibly the most unique attribute of NHP-B is the lophotrichous flagella (5, 8). Birtles et al. (23) refer to “*Candidatus* Odysella thessalonicensis” as being highly motile within the cytoplasm of infected amoebae when examined by light or phase-contrast microscopy, although flagella were not observed by electron microscopy. One documented method of motility that has been described for *Rickettsia rickettsii* is achieved by polymerization of the host cell F-actin, which allows the bacteria to be propelled through the cytoplasm of their host into neighboring cells (17). Although many species of bacteria in the order *Rickettsiales* have means of locomotion that aid in movement from one cell to another, none of the species possess multiple flagella. In 2011, Sasser et al. (24) reported the discovery of 26 genes associated with flagellar assembly, including the hook, filament, and basal body, that were present in the genome of “*Candidatus* Midichloria mitochondrii,” although the bacteria do not have flagella. Approximately 20 *Rickettsiales* genomes have been sequenced, but no additional flagellar genes have been discovered (24). The eight basal flagella in the bacterium are possibly an evolutionary adaptation that has conferred on these bacteria the ability to success-

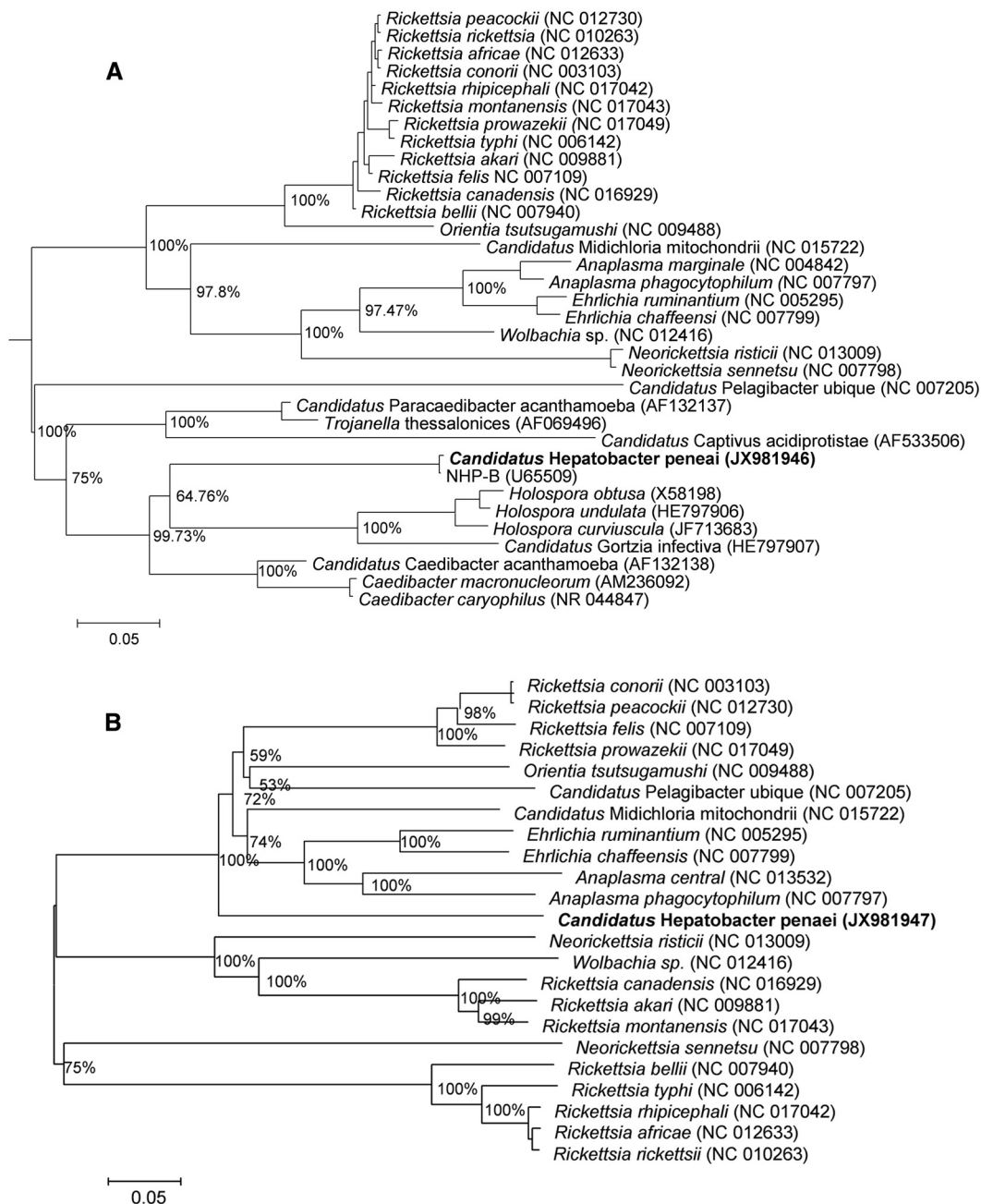
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**FIG 1** Maximum-likelihood phylogenetic tree of the order *Rickettsiales* inferred by using the 16S rRNA (A) and gyrase B (B) gene sequences. The name of the organism in this study is in bold. The accession number is in parentheses after the genus or species name of the bacterium. Numbers at bifurcations represent bootstrap values on 1,000 pseudoreplicates. The bar corresponds to an estimated sequence divergence of 5%.

fully navigate the shrimp's digestive system to allow colonization of the HP, which subsequently causes the pathology seen in NHP. Alternatively, the presence of these flagella also suggests a possible adaptation for locomotion in the aquatic environment where the shrimp host dwells.

Although many techniques have been used over the years to characterize the causative pathological agent of NHP, the obligate intracellular nature of the bacteria has prevented the unequivocal isolation of the strain. Under these constraints, according to Murray and Schleifer (25), we propose the provisional classification of

this bacterium as "*Candidatus Hepatobacter penaei*" gen. nov., sp. nov. Classifying and provisionally naming the bacteria responsible for NHP will help eliminate future confusion with other pathogenic bacteria that can cause similar pathology of the HP in *P. vannamei*.

**Description of "*Candidatus Hepatobacter penaei*" gen. nov., sp. nov.** *Hepatobacter penaei* (He.pa'to.bac.ter. G. n., the liver, N.L. masc. n. bacter, a rod; N.L. masc. n. *Hepatobacter*; pe.nae'i. N.L. n. *Penaeus* a genus of penaeid shrimp; (N.L. gen. n. *penaei* of *Penaeus vannamei*, from which the organism was isolated).

Obligate intercellular pathogen of *Penaeus vannamei*, the Pacific white shrimp, that cannot be cultivated in cell-free media. The geographic range is limited to the western hemisphere. The Gram-negative organism is motile by means of eight flagella located at the basal apex. The bacteria present two morphological variants consisting of a rod-shaped organism (0.25 by 0.9  $\mu\text{m}$ ) and a helical form (0.25 by 2 to 3.5  $\mu\text{m}$ ). Phylogenetic analysis, inferred from 16S rRNA and gyrase B gene sequences, places this bacterium within the class *Alphaproteobacteria*.

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